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For more technical information,
contact NEN Technical Services
at 800-225-1572

CAUTION

Not for use in humans or clinical diagnosis. This product is intended for research or manufacturing use only. It is pharmaceutically unrefined and verification of its suitability for use in humans or as a clinical diagnostic reagent and the compliance with all federal and state laws regulating such applications is solely the responsibility of the purchaser.

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NEN Products
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Boston, Massachusetts 02118



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GeneScreen Plus™

Hybridization Transfer Membrane*

Catalog No. NEF-976

**Protocols for Electrophoretic
and Capillary Transfer of DNA and RNA,
DNA and RNA Hybridization, and
DNA and RNA Rehybridization**

July 1985

**FOR RESEARCH
USE ONLY**



*Method of Use U.S. Patent 4,455,370

INTRODUCTION

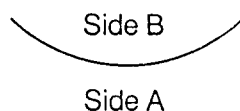
GeneScreenPlus is a second-generation synthetic transfer membrane which was designed to provide the highest sensitivity. This new membrane is positively-charged and supported Nylon 66; it retains the strength of GeneScreen and has the added feature of not requiring baking for fixing nucleic acids onto the membrane.

Its unique construction confers on GeneScreenPlus a natural curl when dry. Wetting eliminates the curl. Both sides of this membrane have excellent binding properties, but different sides are used for different applications. Therefore, it is important to differentiate between the sides to take full advantage of GeneScreenPlus attributes. For the purpose of this discussion, the two sides will be referred to as follows:

Looking at the dry membrane—*

Side A is the convex side

Side B is the concave side



I. TRANSFER OF DNA

The following procedures for the transfer of DNA fragments from electrophoresis gels to GeneScreenPlus and subsequent hybridization have been used extensively in NEN's laboratories with consistent, high quality results. They are reproduced here solely to describe our methodology for the testing and quality control of GeneScreenPlus and may not be universally applicable. These protocols differ from those that are normally used with nitrocellulose.

* Membrane may curl in the opposite direction when wet.

PROCEDURES

Sample and gel preparation: The digestion and electrophoresis of DNA samples can be performed according to a variety of procedures. Do not use sandblasted plates for agarose gels since they cause the agarose to adhere to the membrane, creating background problems.

DNA Transfer: The procedures described have been used by NEN to transfer DNA fragments ranging in size from 75 to 23,000 base pairs from 5–6mm thick agarose gels to GeneScreenPlus. For more efficient transfer of large DNA fragments, the DNA can be "acid nicked" (depurinated) by incubating the gel in 0.25N HCl for 15 minutes at room temperature prior to alkaline treatment (see Section A, Step 1).¹

A. CAPILLARY BLOT PROCEDURE

- (1) Incubate gel in 0.4N NaOH – 0.6M NaCl for 30 minutes at room temperature with gentle agitation to denature DNA.
- (2) Incubate gel in 1.5M NaCl – 0.5M Tris-HCl, pH 7.5, for 30 minutes at room temperature with gentle agitation to neutralize gel.
- (3) Cut GeneScreenPlus to exact size of gel. (Cut membrane between liner sheets; wear gloves). Place a mark on Side B (See Introduction).
- (4) Carefully place the GeneScreenPlus onto the surface of a tray of deionized water. Allow the membrane to wet by capillary action.
- (5) Lay GeneScreenPlus onto a 10 × SSC solution.* Let the membrane soak for approximately 15 minutes.

*10 × SSC: 1.5M sodium chloride – 0.15M sodium citrate. A 10 × SSC solution is used in this procedure to facilitate transfer of DNA from the gel to GeneScreenPlus. The salt is NOT required for efficient binding of DNA to GeneScreenPlus.

- (6) Place a sponge into a tray or glass dish filled with 10 × SSC. The surface of the sponge, when wet, should be larger than the gel. The level of 10 × SSC should be below the top surface of the sponge.†
- (7) Cut two pieces of filter paper (Whatman** 3MM) to the size of the sponge.
- (8) Wet filter paper in 10 × SSC.
- (9) Place the filter paper on the sponge.
- (10) Place gel on filter paper and place gel spacers along each side of the gel.
- (11) Carefully place GeneScreen*Plus* on the gel so that Side B is in contact with the gel. Make sure that no air bubbles are trapped between the gel and the membrane.
- (12) Place 5–6 pieces of dry filter paper (cut to the same size as the gel) on top of the membrane.
- (13) Place 2–3" stack of absorbent paper towels (cut to the same size as the gel) on top of the filter papers.
- (14) Place a small weight on top of the paper towels.
- (15) Allow the transfer to continue for 16–24 hours. Change paper towels frequently. Add more 10 × SSC as needed.
- (16) Carefully remove towels and filter paper without disturbing membrane.
- (17) Wear gloves. Carefully lift membrane away from gel.

†If use of a sponge is not desired, place two pieces of filter paper, pre-wet in 10 × SSC, over an elevated glass plate so that the ends form wicks. Place gel on filter paper and then follow steps 11–20.

**Whatman Limited, England.

- (18) Immerse membrane in an excess of 0.4N NaOH for 30–60 seconds to ensure complete denaturation of immobilized DNA.
- (19) Remove membrane from the NaOH solution and immerse in an excess of 0.2M Tris-HCl, pH 7.5 – 2 × SSC.†
- (20) Place membrane with transferred DNA face up on a piece of filter paper. Allow membrane to dry at room temperature. (THERE IS NO NEED TO FIX DNA ONTO GENESCREEN*PLUS* BY BAKING.)
- (21) Upon drying, the membrane will assume its characteristic curl. To place membrane into the hybridization bag, manually uncurl one end and slide this end into the bag. Once this end is inside the bag, gently slide the rest of the membrane into the bag.

B. ELECTRO-BLOT PROCEDURE

- (1) Incubate gel in 0.4N NaOH for 30 minutes at room temperature with gentle agitation to denature DNA.
- (2) Incubate gel in 12mM Tris – 6mM sodium acetate – 0.3mM EDTA, pH 7.5, for 30 minutes at room temperature to neutralize gel.
- (3) Cut GeneScreen*Plus* to exact size of gel. (Cut membrane between liner sheets; wear gloves). Place a mark on Side B (See Introduction).
- (4) Lay GeneScreen*Plus* onto a solution of 12mM Tris – 6mM sodium acetate – 0.3mM EDTA, pH 7.5. Let the membrane soak for approximately 15 minutes.
- (5) Wet two Scotch-Brite* pads and four pieces of filter paper (Whatman 3MM) in 12mM Tris – 6mM sodium acetate – 0.3mM EDTA, pH 7.5.

†2 × SSC: 0.3M sodium chloride – 0.03M sodium citrate.

*Scotch-Brite is a registered trademark of the 3M Company.

- (6) Place two pieces of filter paper on a Scotch-Brite pad.
- (7) Place gel on top of filter paper. (An alternative procedure is to lay the GeneScreen*Plus* onto the filter paper with Side B facing up. Lay gel on top of the GeneScreen*Plus*. This may help to eliminate air bubbles between the gel and the membrane).
- (8) Carefully place GeneScreen*Plus* on the gel so that Side B is in contact with the gel. Make sure no air bubbles are trapped between the gel and the membrane.
- (9) Place two pieces of filter paper onto the GeneScreen*Plus*.
- (10) Complete the "sandwich" with a second Scotch-Brite pad.
- (11) Insert the "sandwich" of filter paper, gel and GeneScreen*Plus* into a cassette holder and load into a transfer apparatus (any of the commercially available units are suitable) with the GeneScreen*Plus* positioned between the gel and the anode (positive electrode).
- (12) Fill the transfer apparatus with 12mM Tris – 6mM sodium acetate – 0.3mM EDTA, pH 7.5.
- (13) Transfer the DNA fragments electrophoretically from the gel to GeneScreen*Plus*. Electro-blotting conditions will vary depending on the size and type of DNA transferred, as well as on the electrophoretic transfer apparatus that is used.

In our experience, the following conditions have worked well.

- a. Transfer initially at 10 volts (1 volt/cm) for 1 hour at 5°C. In general, small fragments transfer more effectively at lower voltage.

- b. Increase voltage to 40 volts (4 volts/cm) for 2 hours at 5°C to complete transfer of large fragments.

- (14) Remove the membrane and gel from the transfer apparatus.
- (15) Wear gloves. Lift membrane away from gel.
- (16) Rinse membrane in 12mM Tris – 6mM sodium acetate – 0.3mM EDTA, pH 7.5, to remove residual agarose.
- (17) Place membrane with transferred DNA face up on a piece of filter paper. Allow membrane to dry at room temperature. (THERE IS NO NEED TO FIX DNA ONTO GENESCREEN*PLUS* BY BAKING.)
- (18) Upon drying, the membrane will assume its characteristic curl. To place membrane in the hybridization bag, manually uncurl one end and slide this end into the bag. Once this end is inside the bag, gently slide the rest of the membrane into the bag.

C. DOT BLOT PROCEDURE

- (1) Cut GeneScreen*Plus* membrane (while between liner sheets; wear gloves) and filter pad to fit one of the commercially available manifolds.
- (2) Remove liners and, gently lay membrane and filter pad onto 0.4M Tris – HCl, pH 7.5 and soak for 30 minutes.
- (3) Denature DNA in 0.25N NaOH for 10 minutes.
- (4) Chill DNA on ice.
- (5) Dilute DNA to desired concentration in 0.125N NaOH, 0.125 × SSC.*
- (6) Place filter pad and membrane in manifold and clamp tightly.

*0.125 × SSC: 0.01875M Sodium Chloride, 0.001875M Sodium Citrate

- (7) Add diluted DNA to wells of the manifold.
- (8) Allow solution to remain on the membrane without any suction for 30 minutes.
- (9) After 30 minutes, apply a slight suction to manifold for ~ 30 seconds.
- (10) Remove membrane and allow to air dry at room temperature.

II. HYBRIDIZATION OF DNA (Preferred Methods)

A. HYBRIDIZATION AT 65°C

- (1) Prehybridize the membrane by treating it in 10ml of the following solution. ** (See Appendix A for preparation of buffer): 1% SDS, 1M sodium chloride, and 10% dextran sulfate. *** The solution is added to a sealable plastic bag containing the membrane; the plastic bag is sealed and incubated with constant agitation for at least 15 minutes at 65°C.

- (2) Add 0.5-1.0ml of the following solution to bag containing the prehybridization buffer and the membrane:

Denatured salmon sperm DNA ($\geq 100 \mu\text{g/ml}$)^{††} and denatured radioactive probe.^{††} Final concentration of the probe in the bag should be $\leq 10\text{ng/ml}$ ($1-4 \times 10^5\text{dpm/ml}$) for optimum signal-to-background ratio.

Reseal plastic bag and incubate with constant agitation for 6–24 hours at 65°C.

**In our experience, Denhardt's solution and non-homologous DNA are not necessary. Addition of these ingredients will not affect the results. SDS is important for reducing background. If a 1% concentration of SDS is undesirable, the SDS concentration may be reduced. However, we have observed that high background levels result if the SDS concentration drops below 0.5%.

†If buffering is desired, add Tris-HCl, pH 7.5, to a final concentration of 50mM in the solution.

***The dextran sulfate is a product of Pharmacia Fine Chemicals AB, Uppsala, Sweden.

††DNA is denatured in water by heating at 90–100°C for 10 minutes.

- (3) Remove membrane from hybridization solution and wash as follows:

- a. $2 \times 100 \text{ ml}$ of $2 \times \text{SSC}^{\S}$ at room temperature for 5 minutes with constant agitation.
- b. $2 \times 200\text{ml}$ of a solution containing $2 \times \text{SSC}$ and 1.0% SDS at 65°C for 30 minutes with constant agitation.
- c. $2 \times 100\text{ml}$ of $0.1 \times \text{SSC}^{\S\S}$ at room temperature for 30 minutes with constant agitation.

- (4) Place the membrane with the DNA face up on a sheet of filter paper. Allow the membrane to dry at room temperature. (Do not dry if rehybridization is planned; refer to Section IV.)

- (5) Expose and develop autoradiograph as usual.

B. HYBRIDIZATION AT 42°C – FORMAMIDE PROCEDURE

- (1) Prehybridize the membrane by treating it in 10ml of the following solution. *† (See Appendix A for preparation of buffer): 50% Formamide (deionized)** 1% SDS, 1M sodium chloride, and 10% dextran sulfate. The solution is added to a sealable plastic bag containing the membrane; the plastic bag is sealed and incubated with constant agitation for at least 15 minutes at 42°C.

*In our experience, Denhardt's solution and non-homologous DNA are not necessary. Addition of these ingredients will not affect the results. SDS is important for reducing background. If a 1% concentration of SDS is undesirable, the SDS concentration may be reduced. However, we have observed that high background levels result if the SDS concentration drops below 0.5%.

†If buffering is desired, add Tris-HCl, pH 7.5, to a final concentration of 50mM in the solution.

**Formamide is deionized as follows: 100ml of formamide is mixed with 5g of AG 501-X8(D) Mixed Bed Resin, a product of Bio-Rad Laboratories, stirred for 30 minutes at room temperature and filtered to remove resin. For best results, formamide should be prepared fresh daily.

††DNA is denatured in water by heating at 90–100°C for 10 minutes

§ $2 \times \text{SSC}$: 0.3M sodium chloride – 0.03M sodium citrate.

§§ $0.1 \times \text{SSC}$: 0.015M sodium chloride – 0.0015M sodium citrate.

- (2) Add 0.5–1.0ml of the following solution to bag containing the prehybridization buffer and the membrane:

Denatured salmon sperm DNA ($\geq 100 \mu\text{g/ml}$)[†] and denatured radioactive probe.[†] Final concentration of the probe in the bag should be $\leq 10\text{ng/ml}$ ($1-4 \times 10^5\text{dpm/ml}$) for optimum signal-to-background ratio.

Reseal plastic bag and incubate with constant agitation for 6–24 hours at 42°C.

- (3) Remove membrane from hybridization solution and wash as follows:

- a. $2 \times 100\text{ ml}$ of $2 \times \text{SSC}^{\S}$ at room temperature for 5 minutes with constant agitation.
- b. $2 \times 200\text{ ml}$ of a solution containing $2 \times \text{SSC}$ and 1.0% SDS at 65°C for 30 minutes with constant agitation.
- c. $2 \times 100\text{ ml}$ of $0.1 \times \text{SSC}^{\S\S}$ at room temperature for 30 minutes with constant agitation.

- (4) Place the membrane with the DNA face up on a sheet of filter paper. Allow the membrane to dry at room temperature. (Do not dry if rehybridization is planned; refer to Section IV.)

- (5) Expose and develop autoradiograph as usual.

III. HYBRIDIZATION OF DNA (Alternate Method)

- (1) Prehybridize the membrane by treating it in 10ml of the following solution (See Appendix B for preparation of buffer):

^{††}DNA is denatured in water by heating at 90–100°C for 10 minutes.

[§] $2 \times \text{SSC}$: 0.3M sodium chloride – 0.03M sodium citrate.

^{§§} $0.1 \times \text{SSC}$: 0.015M sodium chloride – 0.0015M sodium citrate.

0.2% polyvinyl-pyrrolidone (M.W.40,000), 0.2% ficoll (M.W. 400,000), 0.2% bovine serum albumin, 0.05M Tris-HCl, pH 7.5, 1M sodium chloride, 0.1% sodium pyrophosphate, 1% SDS*, 10% dextran sulfate (M.W. 500,000) and denatured salmon sperm DNA ($\geq 100\text{mg/ml}$).[†]

The solution is added to a sealable plastic bag containing the membrane; the plastic bag is sealed and incubated with constant agitation for at least 6 hours at 65°C.

- (2) Add 0.5–1.0ml of the following solution to bag containing the prehybridization buffer and the membranes.

Denatured salmon sperm DNA ($\geq 100\mu\text{g/ml}$)[†] and denatured radioactive probe.[†] Final concentration of the probe in the bag should be $\leq 10\text{ng/ml}$ ($1-4 \times 10^5\text{dpm/ml}$) for optimum signal-to-background ratio.

Reseal plastic bag and incubate with constant agitation for 6–24 hours at 65°C.

- (3) Remove membrane from hybridization solution and wash as follows:

- a. $2 \times 100\text{ ml}$ of $2 \times \text{SSC}^{**}$ at room temperature for 5 minutes with constant agitation.
- b. $2 \times 200\text{ ml}$ of a solution containing $2 \times \text{SSC}$ and 1.0% SDS at 65°C for 30 minutes with constant agitation.

*In our experience, Denhardt's solution and non-homologous DNA are not necessary. Addition of these ingredients will not affect the results. SDS is important for reducing background. If a 1% concentration of SDS is undesirable, the concentration may be reduced. However, we have observed that high background levels result if the SDS concentration drops below 0.5%.

[†]DNA is denatured in water by heating at 90–100°C for 10 minutes.

^{**} $2 \times \text{SSC}$: 0.3M sodium chloride – 0.03M sodium citrate.

c. $2 \times 100\text{ml}$ of $0.1 \times \text{SSC}^*$ at room temperature for 30 minutes with constant agitation.

- (4) Place the membrane with the DNA face up on a sheet of filter paper. Allow the membrane to dry at room temperature. (Do not dry if rehybridization is planned; refer to Section IV.)
- (5) Expose and develop autoradiograph as usual.

IV. REHYBRIDIZATION OF DNA²

- (1) Do not allow GeneScreenPlus to dry completely. Irreversible binding of probe may result.
- (2) Incubate GeneScreenPlus in 100–200ml of 0.4N NaOH at 42°C for 30 minutes with gentle agitation.
- (3) Remove 0.4N NaOH and incubate GeneScreenPlus in 100–200ml of $0.1 \times \text{SSC}$, 0.1% SDS, 0.2M Tris-HCl, pH 7.5, at 42°C for 30 minutes with gentle agitation.
- (4) Remove membrane, blot between paper towels, and autoradiograph for an appropriate period of time to determine if sufficient probe has been removed.[†] If sufficient probe has not been removed, repeat steps (2) and (3) and check again before proceeding.
- (5) Prehybridize and hybridize as described above.

V. TRANSFER OF RNA

A. FORMALDEHYDE GEL — CAPILLARY BLOT³

(It is strongly recommended that all steps in the following procedure be conducted in a fume hood due to the caustic nature of formaldehyde fumes.)

* $0.1 \times \text{SSC}$: 0.015M sodium chloride — 0.0015M sodium citrate.

[†]In our experience, this procedure removes between ~90–95% of labeled probe.

Gel preparation: for each 100ml of 1% agarose solution needed, add 1.0 g of agarose to 73ml of distilled water and dissolve by boiling. When completely dissolved, let cool at room temperature until the solution temperature reaches 60–65°C. Add 10ml of 120mM Tris – 60mM sodium acetate – 3mM sodium EDTA, pH 7.5 and 16.2ml of 37% formaldehyde (final concentration = 6%); mix and pour gel immediately.

Sample preparation: the RNA should be dissolved in an appropriate volume of a solution containing: 50% formamide (deionized),* 6% formaldehyde and 12mM Tris – 6mM sodium acetate – 0.3mM EDTA, pH 7.5.

After incubating for 15 minutes at 60°C, cool on ice, add tracking dye (e.g. Xylene cyanole-bromophenol blue) and apply sample to gel.

Electrophoresis: Electrophoresis is conducted in the presence of 12mM Tris – 6mM sodium acetate – 0.3mM EDTA, pH 7.5.

RNA TRANSFER—CAPILLARY BLOT (The following procedure has been used to transfer RNA from 4S to 23S from 5–6mm thick agarose gels to GeneScreenPlus.)

- (1) Rinse gel briefly in distilled water to remove excess formaldehyde. (Incubation of gel in 50mM NaOH for 30 minutes may help facilitate transfer of larger RNA, e.g., > 16S. Neutralize the gel in excess Tris buffer, pH 7.0, for 30 minutes.)
- (2) Cut GeneScreenPlus (while between liner sheets; wear gloves) to exact size of gel. Place a mark on Side B. (See Introduction.)
- (3) Wet GeneScreenPlus in distilled water.
- (4) Lay GeneScreenPlus onto a tray or glass dish filled with $10 \times \text{SSC}$ solution.[†] Let the membrane soak for approximately 15 minutes.

*Formamide is deionized as follows: 100ml of formamide is mixed with 5g of AG 501-X8(D) Mixed Bed Resin, a product of Bio-Rad Laboratories, stirred for 30 minutes at room temperature and filtered to remove resin. For best results, formamide should be prepared fresh daily.

[†] $10 \times \text{SSC}$: 1.5M sodium chloride – 0.15M sodium citrate.

- (5) Place a sponge into a tray or glass dish filled with $10 \times$ SSC. ** The surface of the sponge, when wet, should be larger than the gel. The level of $10 \times$ SSC should be below the top surface of the sponge.*
- (6) Cut two pieces of filter paper (Whatman 3MM) to the size of the sponge.
- (7) Wet filter paper in $10 \times$ SSC.**
- (8) Place the filter paper on the sponge.
- (9) Place gel on filter paper and place gel spacers along each side of the gel.
- (10) Carefully place GeneScreen*Plus* on the gel so that side B is in contact with the gel. Make sure that no air bubbles are trapped between the gel and the membrane.
- (11) Place 5–6 pieces of dry filter paper (cut to same size as the gel) on top of the membrane.
- (12) Place 2–3" stack of absorbent paper towels (cut to same size as the gel) on top of the filter papers.
- (13) Place a small weight on top of the paper towels.
- (14) Allow the transfer to continue for 16–24 hours. Change paper towels frequently. Add more $10 \times$ SSC as needed.
- (15) Carefully remove towels and filter paper without disturbing membrane.
- (16) Wear gloves. Carefully lift membrane away from gel.

*If use of a sponge is not desired, place two pieces of filter paper, pre-wet in $10 \times$ SSC, over an elevated glass plate so that the ends form wicks. Place gel on filter paper and then follow steps 10 thru 19.

** $10 \times$ SSC: 1.5M sodium chloride — 0.15M sodium citrate.

- (17) Rinse membrane in $2 \times$ SSC† to remove residual agarose. (Residual agarose on membrane may lead to background problems.)
- (18) Place membrane with transferred RNA face up on a piece of filter paper. Allow membrane to dry at room temperature.
- (19) FORMALDEHYDE REVERSAL
 - a. Bake membrane at 80°C for 2 hours. (Note: This step is required to reverse the formaldehyde reaction.)
 - b. Upon drying, the membrane will assume its characteristic curl. To place membrane into the hybridization bag, manually uncurl one end and slide this end into the bag. Once this end is inside the bag, gently slide the rest of the membrane into the bag.

B. GLYOXAL GEL — CAPILLARY BLOT⁴

Gel preparation: Prepare 1% agarose gel in 12mM Tris – 6mM sodium acetate – 0.3mM EDTA, pH 7.0.

Sample preparation: The RNA should be dissolved in an appropriate volume of a solution containing: 1.0M glyoxal (deionized), 50% DMSO, and 12mM Tris – 6mM sodium acetate – 0.3mM EDTA, pH 7.0.

After incubating for 15 minutes at 50°C ⁵, cool on ice, add tracking dye (e.g. Xylene cyanole-bromophenol blue) and apply sample to gel.

Electrophoresis: Electrophoresis is conducted in the presence of 12mM Tris – 6mM sodium acetate – 0.3mM EDTA, pH 7.0, with buffer recirculation. Without buffer recirculation, change buffer approximately every hour to maintain at a pH less than 8.0.

RNA TRANSFER — CAPILLARY BLOT (The following procedure has been used to transfer RNA from 4S to 23S from 5–6mm thick agarose gels to GeneScreen*Plus*.)

† $2 \times$ SSC: 0.3M sodium chloride – 0.03M sodium citrate.

- (1) Cut GeneScreen*Plus* (while between liner sheets; wear gloves) to exact size of gel. Place a mark on Side B. (See Introduction.)
- (2) Wet GeneScreen*Plus* in distilled water.
- (3) Lay GeneScreen*Plus* onto a 10 × SSC solution.* Let the membrane soak for approximately 15 minutes.
- (4) Place a sponge into a tray or glass dish filled with 10 × SSC. The surface of the sponge, when wet, should be larger than the gel. The level of 10 × SSC should be below the top surface of the sponge.†
- (5) Cut two pieces of filter paper (Whatman 3MM) to the size of the sponge.
- (6) Wet filter paper in 10 × SSC.
- (7) Place the filter paper on the sponge.
- (8) Place gel on filter paper and place gel spacers along each side of the gel.
- (9) Carefully place GeneScreen*Plus* on the gel so that Side B is in contact with the gel. Make sure that no air bubbles are trapped between the gel and the membrane.
- (10) Place 5–6 pieces of dry filter paper (cut to the same size as the gel) on top of the membrane.
- (11) Place 2–3" stack of absorbent paper towels (cut to the same size as the gel) on top of the filter papers.

* 10 × SSC: 1.5M sodium chloride – 0.15M sodium citrate.

† If use of a sponge is not desired, place two pieces of filter paper, pre-wet in 10 × SSC, over an elevated glass plate so that the ends form wicks. Place gel on filter paper and then follow 9–19.

- (12) Place a small weight on top of the paper towels.
- (13) Allow the transfer to continue for 16–24 hours. Change paper towels frequently. Add more 10 × SSC* as needed.
- (14) Carefully remove towels and filter paper without disturbing membrane.
- (15) Wear gloves. Carefully lift membrane away from gel.

REVERSAL OF GLYOXYLATION

METHOD 1.

- (16) Place membrane in a 50mM NaOH solution and incubate for about 15 seconds (20 seconds maximum) to reverse glyoxal reaction.
- (17) Remove membrane from 50mM NaOH and place into solution of 1 × SSC†–0.2M Tris-HCl, pH 7.5. Incubate for approximately 30 seconds.

METHOD 2⁶

- (16) Add membrane to 20mM Tris-HCl, pH 8.0, at 100°C and incubate for 5 minutes.
- (17) Remove membrane from the hot solution with a pair of tweezers.
- (18) Place membrane with transferred RNA face up on a piece of filter paper. Allow membrane to dry at room temperature. There is no need to fix the RNA onto GeneScreen*Plus* by baking.
- (19) Upon drying, the membrane will assume its characteristic curl. To place membrane into the hybridization bag, manually uncurl one end and slide this end into the bag. Once this end is inside the bag, gently slide the rest of the membrane into the bag.

* 10 × SSC: 1.5M sodium chloride – 0.15M sodium citrate.

† 1 × SSC: 0.15M sodium chloride – 0.015M sodium citrate.

C. ELECTROBLOT PROCEDURE

- (1) Cut GeneScreen*Plus* (while between liner sheets; wear gloves) to exact size of gel. Place a mark on Side B. (See Introduction.)
- (2) Lay GeneScreen*Plus* onto a solution of 12mM Tris – 6mM sodium acetate – 0.3mM EDTA, pH 7.5. Let the membrane soak for approximately 15 minutes.
- (3) Wet two Scotch-Brite pads and four pieces of filter paper (Whatman 3MM) in 12mM Tris – 6mM sodium acetate – 0.3mM EDTA, pH 7.5.
- (4) Place two pieces of filter paper on a Scotch-Brite pad.
- (5) Place gel on top of filter paper. (An alternative procedure is to lay the GeneScreen*Plus* onto the filter paper with Side B facing up. Lay gel on top of the GeneScreen*Plus*. This may help to eliminate air bubbles between the gel and the membrane.)
- (6) Carefully place GeneScreen*Plus* on the gel so that Side B is in contact with the gel. Make sure no air bubbles are trapped between the gel and membrane.
- (7) Place two pieces of filter paper onto the GeneScreen*Plus*.
- (8) Complete the “sandwich” with a second Scotch-Brite pad.
- (9) Insert the “sandwich” of filter paper, gel and GeneScreen*Plus* into a cassette holder and load into a transfer apparatus (any of the commercially available units are suitable) with the GeneScreen*Plus* positioned between the gel and the anode (positive electrode).
- (10) Fill the transfer apparatus with 12mM Tris – 6mM sodium acetate – 0.3mM EDTA, pH 7.5.

- (11) Transfer the RNA fragments electrophoretically from the gel to GeneScreen*Plus*. Electro-blotting conditions will vary depending on the size and type of RNA transferred, as well as on the electrophoretic transfer apparatus that is used.

In our experience, the following conditions have worked well.

- a. Transfer initially at 10 volts (1 volt/cm) for 1 hour at 5°C. In general, small fragments transfer more effectively at lower voltage.
- b. Increase voltage to 40 volts (4 volts/cm) for 2 hours at 5°C to complete transfer of large fragments.

- (12) Remove the membrane and gel from the transfer apparatus.

- (13) Wear gloves. Lift membrane away from gel.

(14) REVERSAL REACTIONS

FORMALDEHYDE GEL

- a. Rinse membrane in 12mM Tris – 6mM sodium acetate – 0.3mM EDTA, pH 7.5, to remove residual agarose.
- b. Place membrane with transferred RNA face up on a piece of filter paper. Allow membrane to dry at room temperature.
- c. Bake membrane at 80°C for 2 hours. (Note: This step is required to reverse the formaldehyde reaction.)

GLYOXAL GEL

- a. Place membrane in a 50mM NaOH solution and incubate for approximately 15 seconds to reverse glyoxal reaction.

b. Remove membrane from 50mM NaOH and place into a solution of 1 × SSC* – 0.2M Tris-HCl, pH 7.5. Incubate for approximately 30 seconds. Place membrane with transferred RNA face up on a piece of filter paper. Allow membrane to dry at room temperature. There is no need to fix the RNA onto GeneScreen*Plus* by baking.

(15) Upon drying, the membrane will assume its characteristic curl. To place membrane in the hybridization bag, manually uncurl one end and slide this end into the bag. Once this end is inside the bag, gently slide the rest of the membrane into the bag.

D. DOT BLOT — FORMAMIDE/FORMALDEHYDE PROCEDURE

- (1) Dissolve RNA in appropriate volume of 50% deionized formamide, 6% formaldehyde.
- (2) Incubate for 1 hour at 50°C to denature RNA. Chill on ice.
- (3) Cut GeneScreen*Plus* membrane (while between liner sheets; wear gloves) and filter pad to fit one of the commercially available manifolds. Place a mark on Side B. (See Introduction.)
- (4) Gently lay membrane and filter pad onto deionized water for 15 minutes.
- (5) Dilute RNA to desired concentration in deionized H₂O.
- (6) Place filter pad and membrane with Side B facing up in manifold and clamp tightly.
- (7) Add diluted RNA to wells of the manifold.

*1 × SSC: 0.15M sodium chloride – 0.015M sodium citrate.

- (8) Allow solution to remain on the membrane without any suction for 30 minutes.
- (9) After 30 minutes, apply a slight suction to manifold for 30 seconds.
- (10) Remove membrane and allow to air dry.
- (11) Bake membrane at 80°C for 2 hours to reverse formaldehyde.
- (12) Upon drying, the membrane will assume its characteristic curl. To place membrane in the hybridization bag, manually uncurl one end and slide this end into the bag. Once this end is inside the bag, gently slide the rest of the membrane into the bag.

E. DOT BLOT — GLYOXAL/DMSO PROCEDURE

- (1) Dissolve RNA in appropriate volume of 50% DMSO, 1M deionized glyoxal, 12.5mM sodium phosphate, pH 6.5. (Final pH should be ~9.1.)
- (2) Incubate for 15 minutes at 50°C. Chill on ice.
- (3) Cut GeneScreen*Plus* membrane (while between liner sheets; wear gloves) and filter pad to fit one of the commercially available manifolds. Place a mark on Side B. (See Introduction.)
- (4) Gently lay membrane and filter pad onto deionized water for 15 minutes.
- (5) Dilute RNA to desired concentration in deionized water.
- (6) Place filter pad and membrane with Side B facing up in manifold and clamp tightly.
- (7) Add diluted RNA to wells of the manifold.
- (8) Allow solution to remain on the membrane without any suction for 30 minutes.

- (9) After 30 minutes, apply a slight suction to manifold for 30 seconds.
- (10) Reverse glyoxal by *one* of the following methods:
- Method 1. Heat membrane for 5 minutes at 100°C in 20mM Tris-HCl, pH 8.0.
- Method 2. Soak membrane in 50mM NaOH for 15 seconds. Neutralize membrane in 1 × SSC*, 0.2M Tris-HCl, pH 7.5, for 30 seconds.
- (11) Air dry membrane.
- (12) Upon drying, the membrane will assume its characteristic curl. To place membrane in the hybridization bag, manually uncurl one end and slide this end into the bag. Once this end is inside the bag, gently slide the rest of the membrane into the bag.

VI. HYBRIDIZATION OF RNA (Preferred Methods for all types of transfers)

A. HYBRIDIZATION AT 60°C

- (1) Prehybridize the membrane by treating it in 10ml of the following solution. ** (See Appendix A for preparation of buffer): 1% SDS, 1M sodium chloride, and 10% dextran sulfate.

The solution is added to a sealable plastic bag containing the membrane; the plastic bag is sealed and incubated with constant agitation for at least 15 minutes at 60°C.

*1 × SSC: 0.15M sodium chloride – 0.015M sodium citrate.

** In our experience, Denhardt's solution and non-homologous DNA are not necessary. Addition of these ingredients will not affect the results. SDS is important for reducing background. If a 1% concentration of SDS is undesirable, the concentration may be reduced. However, we have observed that high background levels result if the SDS concentration drops below 0.5%.

- (2) Add 0.5 – 1.0ml of the following solution to bag containing the prehybridization buffer and the membrane:

Denatured salmon sperm DNA ($\geq 100\mu\text{g/ml}$)[†] and denatured radioactive probe.[†] Final concentration of the probe in the bag should be $\leq 10\text{ng/ml}$ ($1-4 \times 10^5\text{dpm/ml}$) for optimum signal-to-background ratio.

Reseal plastic bag and incubate with constant agitation for 6–24 hours at 60°C.

100 ml
10 mg/ml
SS DNA

B. HYBRIDIZATION AT 42°C

- (1) Prehybridize the membrane by treating it in 10ml of the following solution. (See Appendix A for preparation of buffer): 50% formamide (deionized)***, 1% SDS, 1M sodium chloride and 10% dextran sulfate. The solution is added to a sealable plastic bag containing the membrane; the plastic bag is sealed and incubated with constant agitation for at least 15 minutes at 42°C.
- (2) Add 0.5 – 1.0ml of the following solution to bag containing the prehybridization buffer and the membrane:

Denatured salmon sperm DNA ($\geq 100\mu\text{g/ml}$)[†] and denatured radioactive probe.[†] Final concentration of the probe in the bag should be $\leq 10\text{ng/ml}$ ($1-4 \times 10^5\text{dpm/ml}$) for optimum signal-to-background ratio.

Reseal plastic bag and incubate with constant agitation for 6–24 hours at 42°C.

[†]DNA is denatured in water by heating at 90–100°C for 10 minutes.

*** Formamide is deionized as follows: 100ml of formamide is mixed with 5g of AG 501-X8(D) Mixed Bed Resin, a product of Bio-Rad Laboratories, stirred for 30 minutes at room temperature and filtered to remove resin. For best results, formamide should be prepared fresh daily.

C. RECOMMENDED WASHING PROCEDURE

- (1) Remove membrane from hybridization solution and wash as follows:
 - a. $2 \times 100\text{ml}$ of $2 \times \text{SSC}^{**}$ at room temperature for 5 minutes with constant agitation.
 - b. $2 \times 200\text{ml}$ of a solution containing $2 \times \text{SSC}$ and 1.0% SDS at 60°C for 30 minutes with constant agitation.
 - c. $2 \times 100\text{ml}$ of $0.1 \times \text{SSC}^{**}$ at room temperature for 30 minutes with constant agitation.
- (2) Place the membrane with RNA face up on a sheet of filter paper. Allow the membrane to dry at room temperature. (Do not dry if rehybridization is planned; refer to Section VIII.)
- (3) Expose and develop autoradiograph as usual.

VII. HYBRIDIZATION OF RNA (Alternate Method)

- (1) Prehybridize the membrane by treating it in 10ml of the following solution. (See Appendix B for preparation of buffer):

0.2% polyvinyl-pyrrolidone (M.W. 40,000), 0.2% ficoll (M.W. 400,000), 0.2% bovine serum albumin, 0.05M Tris-HCl, pH 7.5, 1M sodium chloride, 0.1% sodium pyrophosphate, 1% SDS,[†] 10% dextran sulfate (M.W. 500,000) and denatured salmon sperm DNA ($\geq 100\mu\text{g/ml}$).^{††}

* $2 \times \text{SSC}$: 0.3M sodium chloride – 0.03M sodium citrate.

** $0.1 \times \text{SSC}$: 0.015M sodium chloride – 0.0015M sodium citrate.

† SDS is important for reducing background. If a 1% concentration is undesirable, the SDS concentration may be reduced. However, we have observed that high background levels result if the SDS concentration drops below 0.5%.

†† DNA is denatured in water by heating at $90\text{--}100^\circ\text{C}$ for 10 minutes.

The solution is added to a sealable plastic bag containing the membrane; the plastic bag is sealed and incubated with constant agitation for at least 1 hour at 60°C .

VIII. REHYBRIDIZATION OF RNA (Preferred Method)

- (1) Do not allow GeneScreen*Plus* to dry completely. Irreversible binding of probe may result.
- (2) Heat 500–600ml of a solution of 0.01% SDS in $0.01 \times \text{SSC}^*$ to boiling.
- (3) Place membrane in the bottom of a clean glass tray, pour about 100–150ml of the hot solution onto the membrane and shake for 2 to 3 minutes. Pour off solution.
- (4) Repeat Step (3) 4 to 5 times.
- (5) Remove membrane, blot between paper towels and autoradiograph for an appropriate period of time to determine if sufficient probe has been removed.
- (6) Prehybridize and hybridize as described above.

IX. REHYBRIDIZATION OF RNA (Alternate Method 1)⁶

- (1) Do not allow GeneScreen*Plus* to dry completely. Irreversible binding of probe may result.
- (2) Bring sterile, distilled water to a boil in a glass tray. Turn off the heat. Immerse the membrane in the hot water for 5–10 minutes. The probe will melt off.
- (3) Remove membrane, blot between paper towels and autoradiograph for an appropriate period of time to determine if sufficient probe has been removed.
- (4) Prehybridize and hybridize as described above.

* $0.01 \times \text{SSC}$: 0.0015M sodium chloride – 0.00015M sodium citrate.

X. REHYBRIDIZATION OF RNA (Alternate Method 2)[†]

- (1) Do not allow GeneScreenPlus to dry completely. Irreversible binding of probe may result.
- (2) Place the membrane into a heat-sealable bag with a volume of wash solution about 3 to 4 times that used for hybridization. The wash solution consists of 10mM Tris, 10mM EDTA (pH 8) and 96% formamide (pH can vary between 7–8). Squeeze out air bubbles and seal the bag.
- (3) Immerse the sealed bag in a 60°C water bath and heat with shaking for 20–30 minutes (30 minutes, maximum). Remove the bag from the bath.
- (4) Remove the membrane from the bag. Rinse the membrane in low salt (1 × SSPE or 2 × SSPE)[†] at room temperature.
- (5) Remove membrane, blot between paper towels and autoradiograph for an appropriate period of time to determine if sufficient probe has been removed. If residual probe is still bound to the membrane, a second wash is likely to remove even this residue. Further, more stringent washes will not remove any more probe.
- (6) Prehybridize and hybridize as described above.

XI. POSSIBLE CAUSES AND REMEDIES FOR HIGH BACKGROUND

(1) Lack of agitation

It is very important to agitate during the prehybridization and hybridization steps. It is extremely important to agitate during washings.

[†]1 × SSPE: 0.18M sodium chloride – 0.01M sodium phosphate, pH 6.8 – 0.001M EDTA.

(2) Probe concentration

- a. In general when dextran sulfate is used, the probe concentration (SA = 10⁷ – 10⁸ cpm/mμg) should be equal to or less than 10ng/ml of solution. Some probes could be as low as 0.5–2.5ng/ml. When dextran sulfate is used the effective concentration of nucleic acids is increased because they are excluded from the volume of solution the polymer occupies. This increases the rate of association of nucleic acid probes by up to 10-fold.¹
- b. When not using dextran sulfate, the optimal probe concentration (SA = 10⁷ – 10⁸ cpm/mμg) has been found to be 25–40ng/ml of solution.
- c. For genomic blots, higher concentrations of probe may be useful. However, this will definitely contribute to background. Therefore, when high probe concentrations are used, it is recommended that the probe be purified.

(3) Probe purification

It is important to purify the probe either by column chromatography or using NENSORB™20 Nucleic Acid Purification Cartridge to remove unincorporated nucleotides, proteins, etc. It is always advisable to clean the probe when working with single copy or low copy number genes.

(4) Residual agarose on membranes

If the membranes are not rinsed after the transfer, residual agarose will cause a fuzzy background to develop.

(5) SDS

- a. Omission of SDS from the prehybridization step causes high background. The concentration can be reduced to 0.5%, but the lower the SDS concentration the higher the background.
- b. Omission of SDS from the washing solution can also cause high background.
- c. If the DNA is UV-fixed to the membranes, the SDS concentration can be as high as 7%.

(6) Dextran sulfate

If dextran sulfate is used, then it must be included in both the prehybridization and hybridization steps. Using dextran sulfate only during the hybridization causes very high and splotchy background patterns.

(7) Washing

- a. More stringent washings may be needed. This is achieved by increasing both the temperature and washing times.
- b. For colony or plaque lifting, stringent washing of the membrane *after* the polynucleotide is fixed is recommended. Wash filters in 3 × SSC,*0.1% SDS at 65°C for 16–20 hours. Use a large volume of buffer and make several changes. The early changes of buffer will smell like bacteria. After washing, the filters may be stored dry at 4°C or used immediately.

(8) The membranes should always be handled with gloves to avoid contamination of RNase, proteins, etc.

(9) Static electricity

Spots all over film may be due to static electricity. This is characterized by small lightning-like marks or by small dark spots which appear fuzzy around the edges.

(10) Water quality

The quality of the distilled water influences background. The intensity of the background is directly related to the organic content of the water.

(11) Dirty glassware

Residual detergent or dirt on the glassware can contribute to severe background problems.

XII. REFERENCES

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*3 × SSC: 0.45M sodium chloride – 0.045M sodium citrate.

XIII. APPENDICES

APPENDIX A

The following procedure is a convenient way to prepare the prehybridization buffer for preferred methods.

- (1) Prepare a 10% SDS solution.
- (2) Prepare a 50% dextran sulfate solution.
- (3) To prepare a 10ml solution for prehybridization, add the following to a centrifuge tube:

	7ml H ₂ O or
7ml	5ml formamide + 2ml H ₂ O
2ml	50% dextran sulfate
<u>1ml</u>	10% SDS
10ml	

Mix by inversion. Place tube in a 42°C (or 65°C if minus formamide) water bath for 10–15 minutes. Add 0.58g of sodium chloride to the tube and mix by inversion. Place tube back into the water bath for 10–15 minutes.

- (4) Remove tube from water bath and pour solution into the bag containing GeneScreen*Plus*. Carefully squeeze out air bubbles, seal bag and place in water bath.

APPENDIX B

The following procedure is a convenient way to prepare the prehybridization buffer for alternate method.

- (1) Prepare a 10% SDS solution.
- (2) Prepare a 50% dextran sulfate solution.

- (3) Prepare a 5 × “P” Buffer:

1% bovine serum albumin, 1% polyvinyl-pyrrolidone (M.W. 40,000), 1% ficoll (M.W. 400,000), 250mM Tris-HCl, pH 7.5 and 0.5% sodium pyrophosphate.

- (4) To prepare a 10ml solution for prehybridization, add the following to a centrifuge tube (Tube A):

4ml	H ₂ O
2ml	5 × “P” Buffer
2ml	50% dextran sulfate
<u>1ml</u>	10% SDS
9ml	Total

Mix by inversion. Place tube in a 65°C water bath for 10–15 minutes. Add 0.58g of sodium chloride to the tube and mix by inversion. (The solution at this point will be cloudy gray.) Place tube back into the 65°C water bath.

- (5) Add 1ml of 1mg/ml salmon sperm DNA to a second centrifuge tube (Tube B). Place tube in boiling water bath for 10 minutes. Cool on ice for 5–10 minutes.
- (6) Remove Tube A from water bath and pour solution into Tube B containing denatured salmon sperm DNA. Mix by inversion.
- (7) Pour solution into the bag containing GeneScreen*Plus*. Carefully squeeze out air bubbles, seal bag and place in water bath.